Electronic Supplementary Information

A target-triggered exponential amplification-based DNAzyme biosensor for ultrasensitive detection of folate receptors

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MATERIALS AND METHODS

Materials. All the oligonucleotides used in this research (Table S1) were synthesized by TaKaRa Bio. Inc. (Dalian, China). The exonuclease I (Exo I), exonuclease III (Exo III), nicking endonuclease (Nt.BstNBI), NEB buffer I, NEB buffer III, Vent (exo-) polymerase, deoxynucleotide triphosphates (dNTPs) and thermopol reaction buffer were obtained from New England Biolabs (Beverly, MA, USA). Bovine serum albumin (BSA) and the human serum were purchased from Dingguo Biotech. Co. (Beijing, China). Thrombin, immunoglobulin G (IgG), folate receptor (FR), folic acid (FA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS) and protoporphyrin IX zinc (II) (ZnPPIX) were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). SYBR Green I (10000×) was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). SYBR Gold (10000×) was purchased from Invitrogen (CA, USA). Magnesium chloride (MgCl₂),...
phosphate-buffered saline (PBS, pH 7.4), trizma hydrochloride-EDTA (pH 7.0), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES) and sodium chloride (NaCl) were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). Dialysis membranes with the molecular mass cut-off ranging from 0.5 kDa to 1 kDa were purchased from Spectra-Pore (Rancho Dominguez, CA, USA). All other chemicals were of analytical grade and used without further purification. Ultrapure water was obtained from a Millipore filtration system. The protoporphyrin IX zinc (II) solution was prepared with dimethysulfoxide (DMSO) as the solvent and stored at −20 °C in the dark.

Table S1. Sequences of the Oigonucleotides*

<table>
<thead>
<tr>
<th>note</th>
<th>sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>hairpin probe</td>
<td>TTA ACA CTG TCT GGG AGT CAA GAT TAA CAC TGT CTG</td>
</tr>
<tr>
<td></td>
<td>GGA GTC AAG AAT AAC CCA TCC CGC CCA ACC CTC TTG</td>
</tr>
<tr>
<td></td>
<td>ACT CCC AGA CAG TGT TAA TCT TGA CTC CCA GAC AGT GTT</td>
</tr>
<tr>
<td></td>
<td>AA-NH₂</td>
</tr>
<tr>
<td>template</td>
<td>ATA ACC CAT CCC GCC CAA CCC TCT TGA CTC CCA GAC AGT</td>
</tr>
<tr>
<td></td>
<td>GTT AAT CTT GAC TCC CAG ACA GTG TTA A-NH₂</td>
</tr>
<tr>
<td>DNAzyme</td>
<td>GGG TTG GGC GGG ATG GGT TAT</td>
</tr>
<tr>
<td>trigger</td>
<td>TTA ACA CTG TCT GGG AGT CAA GA</td>
</tr>
</tbody>
</table>

*The underlined regions symbolize the recognition sequences of Nt.BstNBI nickase. The underlined bold regions symbolize the sequences that will be nicked by Nt.BstNBI nickase upon the formation of DNA duplexes.
Preparation of Cell Extracts. The A549 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37 °C in a humidified chamber containing 5% CO₂. In the exponential phase of growth, the cells were collected for extraction of active proteins with a membrane protein extraction kit (BSP049, Sangon, Shanghai, China). The obtained membrane protein was aliquoted and stored at −20 °C. The protein concentrations were quantified by a modified BCA protein assay kit with bovine serum albumin as the standard (SK3051, Sangon, Shanghai, China).

Conjugation of folic acid with NH₂-Modified DNA and Subsequent Exonucleases Digestion.

Firstly, 1 μM 3'-NH₂-modified hairpin probes were incubated in a buffer containing 1.5 mM MgCl₂ and 10 mM Tris–HCl (pH 8.0) at 95 °C for 5 min, followed by cooling to 37 °C for 30 min to make the probes perfectly fold into hairpin structures. Then 0.5 mL of 1 μM amino-modified hairpin probes was mixed with 0.5 mL of FA solution (0.5 M) in the presence of cross-linking agents which contained 10 μL of EDC (1 mM) and 10 μL of NHS (5 mM), and incubated at 37 °C for 2 h in the dark. The excess folic acid was removed by dialysis using a membrane with a molecular weight cutoff of 1000 Da against the phosphate buffer solution (PBS, pH 7.4). The dialysis was performed for 3 days in the dark with the changing of fresh PBS every 4 h. Then FR at various concentrations was added into the dialyzed solution and incubated at 37 °C for 1 h in the dark. At last, the digestion reaction was performed in the presence of 50 unit Exo III, 20 unit Exo I, and 2 μL of 1× NEB buffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.0), followed by incubation at 37 °C for 1 h. The digestion reaction was terminated by heating
at 95 °C for 10 min.

**Target-Triggered Isothermally Exponential Amplification Reaction.** The FR-triggered isothermally exponential amplification reaction system (20 µL) contains 10 µL of digestion solution, 100 nM template, 2 µL of 10× thermopol reaction buffer (20 mM Tris−HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8), 2 µL of 10× Nt.BstNBI buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.9), 0.5 µL of dNTPs (200 mM each), 1 unit Vent (exo-) DNA polymerase and 8 unit Nt.BstNBI nicking enzyme. The FR-triggered EXPAR was performed at 55 °C for 30 min, and then the reaction was terminated by heating at 85 °C for 10 min.

**Real-Time Fluorescence Measurement and Gel Electrophoresis.** The real-time fluorescence measurement of FR-triggered EXPAR was performed in a BIO-RAD CFX connect™ Real-Time system (Singapore) with 1× SYBR Green I as the fluorescent indicator, and the fluorescence intensity was monitored at the intervals of 30 s. The 12% nondenaturing polyacrylamide gel electrophoresis (PAGE) was used to analyze the products of EXPAR in 1× TBE buffer (9 mM Tris-HCl, pH 7.9, 9 mM boric acid, 0.2 mM EDTA) with 1× SYBR Green I as the fluorescent indicator. The stained gel was scanned by a Kodak Image Station 4000 MM (Rochester, NY, USA).

**Measurement of Fluorescence Spectra.** The 10 µL of amplification products were added to 200 µL of ZnPPIX reaction solution (20 mM HEPES, pH 7.0, 150 mM NaCl, 5 µM ZnPPIX), and
incubated at room temperature for 30 min in the dark. The fluorescence spectra were recorded by a Hitachi F-4600 spectrometer (Tokyo, Japan) at the excitation wavelength of 420 nm.

SUPPLEMENTARY RESULTS

Real-Time Fluorescence Monitoring of Target-Triggered Isothermally Exponential Amplification. To validate the feasibility of the proposed method for FR assay, we performed the real-time fluorescence measurement with SYBR Green I as the fluorescent indicator. In the presence of FR, the binding of FR with FA protects the hairpin probes from being digested by Exo III and Exo I, and the protected hairpin probes can be nicked by the nicking enzyme to initiate the amplification reaction in the presence of DNA polymerase and endonuclease. As shown in Fig. S1A, in the presence of 1 nM FR, the fluorescence intensity increases in a sigmoidal fashion as the amplification products are converted from single-stranded to partially and completely double-stranded DNAs (Fig. S1A, red line). However, in the absence of FR, the hairpin probes are completely digested by Exo III and Exo I, and no significant fluorescence signal is observed (Fig. S1A, blue line). We further analyzed the amplification products using the non-denaturing polyacrylamide gel electrophoresis (PAGE). As shown in Fig. S1B, a well-defined band of DNAzyme (21 nt) is observed in the presence of 1 nM FR (Fig. S1B, lane 2), while no distinguishable band of DNAzyme is observed in the absence of FR (Fig. S1B, lane 3).
Fig. S1 (A) Real-time fluorescence monitoring of target-triggered EXPAR with SYBR Green I as the fluorescent indicator in the presence (red line) and in the absence (blue line) of 1 nM FR. (B) Nondenaturating PAGE analysis of EXPAR products. Lane 1 is the DNA marker. Lanes 2, 3 and 4 represent the products in the presence of 1 nM FR, in the absence of FR, and in the presence of synthetic DNAzymes, respectively.

Optimization of Experimental Condition. To avoid the false positivity caused by the residual FA-linked hairpin probes which are neither bound by FR nor hydrolyzed by Exo III and Exo I, we optimized the digestion time of Exo III and Exo I using SYBR Gold as the fluorescent indicator. SYBR Gold exhibits strong fluorescence in the presence of not only double-stranded DNA but also single-stranded DNA and RNA. As shown in Fig. S2A, the value of $F/F_0$ decreases rapidly with the digestion time from 0 to 30 min, and levels off beyond the digestion time of 45 min ($F$ and $F_0$ are the fluorescence intensity of SYBR Gold in the presence and in the absence of hairpin probes, respectively). To ensure the complete digestion of free hairpin probes, the digestion time of 60 min is used in the subsequent research.

To prevent the non-specific amplification, we further optimized the reaction time of EXPAR. As shown in Fig. S2B, the value of $F/F_0$ increases with the reaction time from 0 to 30 min and then reaches the plague ($F$ and $F_0$ are the fluorescence intensity of ZnPPIX in the presence and in
the absence of FR, respectively). Thus, the reaction time of 30 min is used for EXPAR in the subsequent research.

The cooperation of vent (exo-) polymerase and Nt.Bst NBI nickase has crucial effect on the amplification efficiency.\textsuperscript{5,6} With a fixed concentration of Nt.Bst NBI nickase (0.4 U/\mu L, equivalent to 8 U in this research) as reported previously,\textsuperscript{7} we investigated the influence of vent (exo-) polymerase upon the ratio of fluorescence signal \((F/F_0)\), where \(F\) and \(F_0\) are the fluorescence intensity in the presence and in the absence of FR, respectively. As shown in Fig. S2C, the value of \(F/F_0\) increases with the increasing amount of vent (exo-) polymerase from 0.4 U to 1 U, followed by the decrease beyond the amount of 1 U. Thus, 1 U vent (exo-) polymerase is used in the subsequent research. We further investigated the influence of Nt.Bst NBI nickase upon the value of \(F/F_0\) at a fixed amount of vent (exo-) polymerase (1 U). As shown in Fig. S2D, the value of \(F/F_0\) increases with the increasing amount of Nt.BstNBI nickase from 6 U to 8 U, and reaches the maximum value at 8 U. Therefore, 8 U Nt.BstNBI nickase is used in the subsequent research.

To optimize the incubation time for ZnPPIX binding to DNAzyme, a synthesized DNAzyme was used for the production of fluorescent ZnPPIX/G-quadruplex structures.\textsuperscript{8} As shown in Fig. S2E, the value of \(F/F_0\) increases with the incubation time, and levels off at 30 min (\(F\) and \(F_0\) are the fluorescence intensity in the presence and in the absence of DNAzyme, respectively). Thus, the incubation time of 30 min is used for ZnPPIX binding to DNAzyme in the subsequent research.

We further optimized the ZnPPIX concentration. As shown in Fig. S2F, the value of \(F/F_0\) increases with the increasing concentration of ZnPPIX from 0.1 \mu M to 5 \mu M, followed by the decrease beyond the concentration of 5 \mu M. Therefore, 5 \mu M ZnPPIX is used in the subsequent
Fig. S2. (A) Variance of the $F/F_0$ value with the digestion time of Exo III and Exo I. $F$ and $F_0$ are the fluorescence intensity of SYBR Gold in the presence and in the absence of 1 nM hairpin probes, respectively. (B) Variance of the $F/F_0$ value with the reaction time of EXPAR. $F$ and $F_0$ are the fluorescence intensity in the presence and in the absence of 1 nM FR, respectively. (C) Variance of the $F/F_0$ value with the amount of Vent (exo-) DNA polymerase at a fixed amount of Nt.BstNBI nickase (8 U). $F$ and $F_0$ are the fluorescence intensity in the presence and in the absence of 1 nM FR, respectively. (D) Variance of the $F/F_0$ value with the amount of Nt. BstNBI nickase at a fixed amount of Vent (exo-) DNA polymerase (1 U). $F$ and $F_0$ are the fluorescence intensity in the presence and in the absence of 1 nM FR, respectively. (E) Variance of the $F/F_0$ value with the incubation time for ZnPPIX binding to DNAzyme. $F$ and $F_0$ are the fluorescence intensity in the presence and in the absence of 1 nM FR, respectively.
intensity in the presence and in the absence of 1 μM DNAzyme, respectively. (F) Variance of the $F/F_0$ value with the concentration of ZnPPIX. $F$ and $F_0$ are the fluorescence intensity in the presence and in the absence of 1 μM DNAzyme, respectively. Error bars show the standard deviations of three experiments.

**REFERENCE**


